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Glucose-Induced Secretion of *Trichoderma reesei* Xylanases

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To produce two xylanases with *Trichoderma reesei* grown on glucose, recombinant strains which carry either the *xyn1* or the *xyn2* (xylanase I and II [XYN I and XYN II]-encoding) structural genes under the expression signals of the homologous *pki1* (pyruvate kinase-encoding) gene were constructed. The two types of transformants secreted XYN I or II, respectively, during growth on glucose, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunostaining. The corresponding specific xylanase activities of the best transformants on glucose were 76 and 145 U/mg of protein for XYN I and XYN II, respectively, as opposed to that obtained by the parent strain (26 U/mg of protein). When related to the amount of biomass formed, however, they produced only about 4 to 5 U/g, in contrast to much higher activities (10 to 12 U/g) during growth on xylan. The ultrastructural location of XYN II in the transformant strain producing the highest constitutive XYN II formation (ATX2-12) was investigated by immunoelectron microscopy and compared with that in the wild-type strain growing on xylan. Cell extracts from both types of transformants grown on glucose exhibited a higher intracellular xylanase activity than did the parent strain grown on xylan. By using electron microscopy and immunogold labelling, XYN II was detected in the endoplasmic reticulum, Golgi-like vesicles, secretory vesicles, vacuoles, and cell walls. The immunolabel in the vacuoles was detected preferentially in subapical cells. When a recombinant strain which expressed *xyn2* from the *pki1* promoter was compared with the parent strain during growth on xylan, the former exhibited a less proliferated endoplasmic reticulum and a smaller number of secretory vesicles; however, a higher density of labelling was observed. The relationship of these findings to the efficacy of protein secretion during growth on glucose is discussed.

Selected filamentous fungi are excellent producers of a variety of hydrolytic enzymes. *Trichoderma reesei* is a particular prominent example of this (33) and is well known for its formation of cellulases and hemicellulases in very large amounts (up to 30 g/liter [6]). While it is unlikely that this yield may be further increased, the composition of the enzyme mixture secreted may not always be ideally suited for industrial application. For instance, application in pulp and paper technology requires xylanases that are almost free of cellulases, since the latter affect the quality of the product (3). Although the formation of cellulases and xylanases can be differentially triggered by manipulating the growth medium, formation of either enzyme cannot be completely avoided (7, 15, 16, 22, 27). There is therefore a need to alter the formation of individual enzymes by other means. Gene deletion and gene replacement have been carried out to this end (32), but since the cellulase enzyme system consists of several components (18), this is a tedious approach. Production of individual enzymes in response to differentially regulated expression signals may be a more convenient strategy.

One such approach would be the use of promoters which trigger gene expression on glucose, since cellulase formation is repressed under these conditions (19). This would have the additional advantage that enzyme production occurs in rapidly growing cells on inexpensive substrates such as hydrolyzed starch. While this principle has been demonstrated successfully in yeast cells (8) and in the basidiomycete *Phanerochaete chrysosporium* (25a), attempts to produce enzyme in filamentous fungi were rather unsuccessful (5, 26). The reason for this is unknown. Since the genes whose promoters were used are strongly transcribed, it is possible that other factors limit enzyme secretion during growth on glucose (17).

One of these factors could be the capacity of the secretory pathway on glucose. The biochemical understanding of the secretory pathway in filamentous fungi is still in its infancy (for a review, see reference 24). Previous evidence pointed to the existence in *T. reesei* of two secretory pathways, one constitutive and one inducible (12). Organelles involved in the latter have been shown to accumulate in the hypersecretory mutant strain *T. reesei* RUT C-30 (9–12).

Besides their industrial importance, as stressed above, xylanases, particularly xylanase II (XYN II), of *T. reesei* may be used as a model to study protein secretion by *T. reesei*, since XYN II is not glycosylated (38) and is not retained by the cell wall to any major extent. Also, monoclonal antibodies for its detection by immunoelectron microscopy are available (20,

TABLE 1. *T. reesei* strains used in this study

Strain	Genotype[a]	Source or reference
QM 9414	Mutant of wild-type QM6a	25
TU-6	Δ*pyr4*	13
TU-6 (*pyr4*$^+$)	*pyr4* multicopy (TU-6 complemented)	13
ATX1-4	TU-6, *pki1*$_p$:*xyn1 pyr4*$^+$	This study
ATX1-5	TU-6, *pki1*$_p$:*xyn1 pyr4*$^+$	This study
ATX2-12	TU-6, *pki1*$_p$:*xyn2 pyr4*$^+$	This study
ATX2-14	TU-6, *pki1*$_p$:*xyn2 pyr4*$^+$	This study

[a] The subscripts *p* and *t* indicate 5′ noncoding (promoter) and 3′ noncoding (terminator) sequences, respectively.

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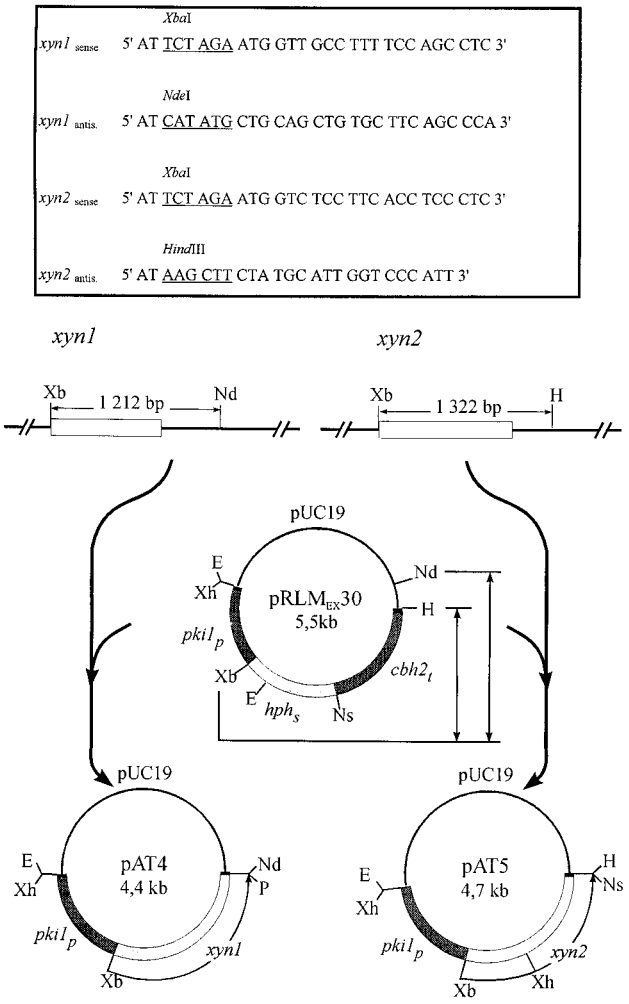

FIG. 1. Structures of plasmids pAT4 and pAT5, used in this study to transform *T. reesei* for constitutive *xyn1* and *xyn2* formation, and of the primers used to amplify the respective *xyn1*- and *xyn2*-coding regions by PCR. Abbreviations: Xb, *Xba*I; Nd, *Nde*I; H, *Hin*dIII; E, *Eco*RI; Xh, *Xho*I; Ns, *Nsi*I.

34). Here we report the results of studying the glucose-induced production of *T. reesei* xylanases by fusing their coding sequences downstream of the expression signals of the homologous pyruvate kinase (*pki1*) promoter (30). We also report some aspects of XYN II secretion under these conditions.

MATERIALS AND METHODS

Strains, transformation, and growth conditions. The *T. reesei* strains used in this study are listed in Table 1. They were maintained on malt agar (containing 1 mM uridine for *T. reesei* TU-6 [13]) and subcultured monthly. Transformants were obtained by cotransformation, as described previously (14), with plasmid pFG1, which carries the homologous *pyr4* (previously termed *pyrG*) gene as a selectable marker. *Escherichia coli* JM109 was used for cloning (39).

For growth and xylanase formation, the strains were cultivated in 1-liter wide-mouth Erlenmeyer flasks containing 200 ml of medium (25) with either glucose or beechwood xylan (Lenzing AG, Lenzing, Austria) as a carbon source (final concentration, 1% [wt/vol]). The flasks were incubated for up to 72 h at 28°C with stirring at 200 rpm.

Plasmids and manipulation of DNA. Plasmids pFG1 (14) and pLMRS3 (23) (see below) were obtained from our department stock. To construct pAT4 and pAT5 (Fig. 1), bearing the prepropetide-encoding DNA sequences of *xyn1* and *xyn2* under the regulation of the *pki1* promoter, the following strategy outlined in Fig. 1 was used. Fragments of *xyn1* (1,212 bp) and *xyn2* (1,322 bp), containing the complete coding sequence and several hundred base pairs of the respective 3′ noncoding sequences, were amplified by PCR. The respective oligonucleotides were designed according to published sequences (35) and constructed in such a way that they included appropriate restriction sites to facilitate subsequent cloning. Thirty cycles, each consisting of 1 min of denaturation at 94°C, 2 min of annealing at 56°C, and 3 min of DNA synthesis at 72°C, were performed. The PCR products were separated by agarose gel electrophoresis, eluted from the gel with a Qiagen kit, and ligated into pLMRS3 (23), which contains the *pki1* promoter upstream of the 3′ noncoding sequences of the homologous *cbh2* (cellobiohydrolase II-encoding) gene, separated by a 5′-*Xba*I-*Sal*I-*Nho*I-3′ cloning site, and which was previously cut with *Xba*I-*Nde*I or *Xba*I-*Hin*dIII. This cleavage results in excision of the *cbh2* 3′ noncoding sequences, which are replaced by ligation of the respective *xyn1* and *xyn2* fragments, yielding pAT4 and pAT5, respectively. The two vectors were verified by double-strand sequencing, and all the restriction sites generated were tested by restriction enzyme analysis.

DNA techniques. *T. reesei* chromosomal DNA was isolated as described previously (14). Double-stranded DNA sequencing was carried out by the dideoxyribonucleotide chain termination method with ^{35}S-dATP (29), with specific oligonucleotides as primers in addition to the universal and the reversed universal M13 primers. All other recombinant DNA techniques were carried out as described previously (28).

Cell extracts. To prepare cell extracts, mycelia were harvested on a Buchner funnel, washed with ice-cold tap water, and blotted dry between filter paper sheets. They were then suspended in 50 mM citrate buffer (pH 5.0) (to give 10 ml/g [wet weight]) and sonicated in an ice bath with a Branson sonifier (15 times for 30 s each, with intermittent 2-min cooling periods). The homogenate was centrifuged at 10,000 × *g* (15 min at 4°C), and the supernatant (typically containing 2 to 4 mg of protein per ml) was kept for enzyme activity determinations. Activities were usually assayed within 3 h after preparation of the extract.

Enzyme assays. The xylanase activity was assayed as described previously (35), with Lenzing xylan (Lenzing AG) as the substrate. Protein concentrations in the culture filtrate were determined by the dye-binding procedure (2).

To assay xylanase activity in the presence of reducing sugars, 5 μl from the culture filtrate was spotted onto 0.8% (wt/vol) agarose gels on microscopic slides containing 0.5% (wt/vol) Lenzing xylan in 50 mM acetate buffer. The gels were incubated at 50°C for 60 min. Thereafter, they were soaked (15 min) in 1 M NaCl, incubated with 0.1% (wt/vol) Congo red (10 min), and washed with 1 M NaCl until the hydrolytic halos became clearly visible. Xylanase samples of known activity were used as controls, and the activity was calculated from a calibration curve of purified XYN I or XYN II, respectively, versus clearing-zone diameter. Purified XYN I and XYN II were obtained as described previously (35).

Electrophoretic techniques. For specific detection of xylanase isoenzymes in

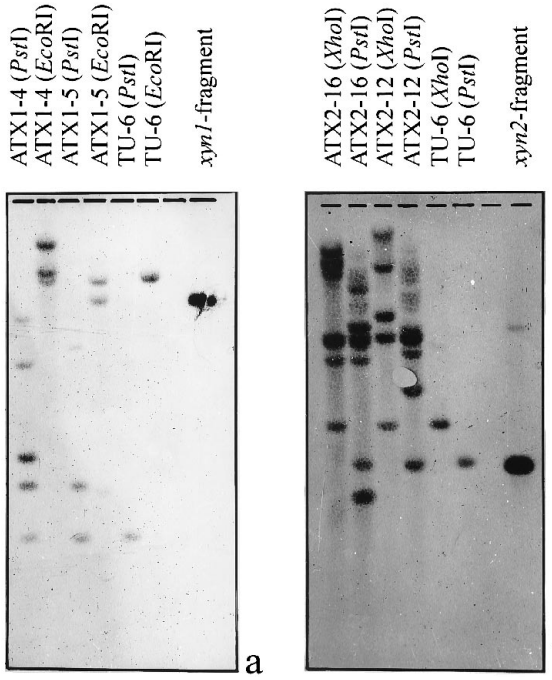

FIG. 2. Southern blot analysis of some strains of *T. reesei* transformed with pATX1 (a) and pATX2 (b). (a) DNA was cleaved with *Pst*I or *Eco*RI. The right-hand lane contains an intact 1,700-bp *xyn1* fragment. A 280-bp *xyn1* fragment was used as a probe. (b) DNA was cleaved with *Xho*I or *Pst*I. The right-hand lane contains a 5-kb fragment of *xyn2*. Hybridization was carried out with a 560-bp *xyn2* fragment as a probe.

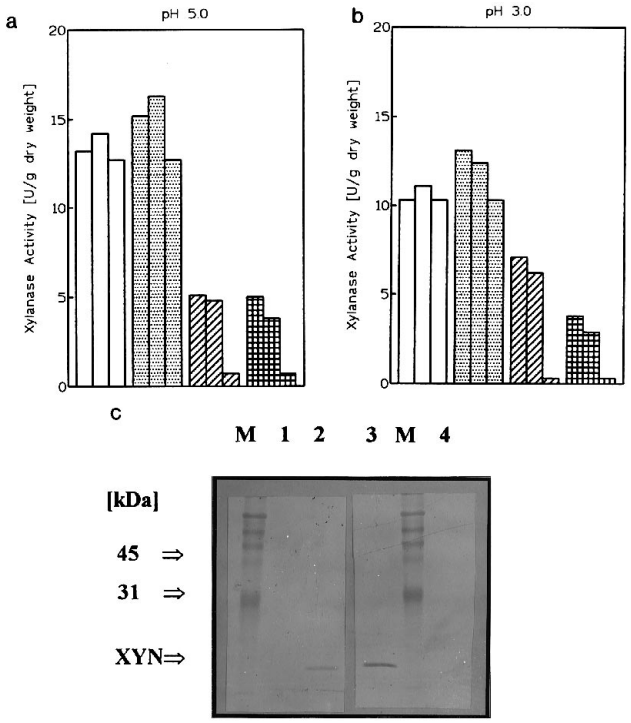

FIG. 3. (a and b) Xylanase activity at pH 5 and 3 upon growth of the recombinant strains in Fig. 2 on xylan (48 h) and glucose (36 h) as the carbon source. *T. reesei* TU-6, complemented to uridine prototrophy by transformation with pFG1, served as a control in all cases. The numbers in panel a indicate xylanase activities, assayed at pH 5.0 after growth on xylan (from left to right, ATX1-4, ATX1-5, TU-6, ATX2-16, ATX2-12, and TU-6) or glucose (ATX1-4, ATX1-5, TU-6, ATX2-16, ATX2-12, and TU-6). The numbers in panel b indicate essentially the same strains at pH 3.0. (c) Immunological identification of XYN I and XYN II on Western blots prepared from glucose-grown cultures of ATX1-4 (lane 2), ATX2-12 (lane 3), and TU-6 (lanes 1 and 4). M indicates prestained marker proteins; the sizes of two of these are given on the left. Lanes 1 and 2 were reacted with the monoclonal antibody against XYN I, and lanes 3 and 4 were reacted with the monoclonal antibody against XYN II. The bottom arrow on the side of the electropherogram indicates the position of intact 19-kDa XYN I and 21-kDa XYN II. Comparable aliquots of the culture filtrate (150 μl; precipitated with 2 volumes of ethanol and redissolved in 15 μl of SDS-PAGE sample buffer) were applied to individual tracks.

the supernatant, samples from the culture broth were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21) and then to Western blotting (immunoblotting) to nitrocellulose (36) and immunological detection as described previously (34). Monoclonal antibodies were used to detect xylanase I (S. Sowka, unpublished) and xylanase II (34).

Immunoelectron microscopy. Mycelia harvested after 72 h of growth on either glucose or xylan in shake flasks and the mycelia of the center and periphery of 72-h-old colonies on solid medium were prepared for electron microscopy as described previously (20). Ultrathin sections were examined under a JEM 100 C transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 keV. Quantitation of immunogold particles in individual compartments was done by counting 100 different sections per sample. The data were statistically treated with the EPISTAT software (Statistical Package Version 3.0, 1984, program data one).

RESULTS

Preparation of *T. reesei* strains producing XYN I or XYN II on glucose. We have prepared plasmid vectors based on pUC19, which contain the genes encoding the two major xylanases of *T. reesei* (*xyn1* and *xyn2* [35]) under the influence of *pki1* (pyruvate kinase-encoding) gene. The respective vectors, pAT4 and pAT5, were introduced into *T. reesei* TU-6 by cotransformation with pFG1 (complementing *pyr4* auxotrophy) as the selection marker. Approximately 30% of the *pyr4*-complemented strains also contained pAT4 or pAT5 (data not shown). Twenty of each class were subsequently tested for mitotic stability; this finally yielded four and three stable ATX1-(*pki1*:*xyn1*)- and ATX2-(*pki1*:*xyn2*)- transformants, respectively. Southern blot analysis of these transformants shows that in these strains, the xylanase genes had become integrated into the fungal genome in multiple copies and at multiple loci (Fig. 2). They were grown in liquid medium, with glucose or xylan as the carbon source, and the culture filtrates were analyzed by assaying xylanase activity and by immunological detection on Western blots (Fig. 3). Enzyme determination was carried out at pHs 3 and 5 to account for the differences in the optimal pH of XYN I and XYN II (35). All transformants showed at least some xylanase activity on glucose. The specific activities of both XYN I and XYN II on glucose were considerably higher than on xylan, because of the much lower total protein contents in the culture fluids (Table 2). When related to the amount of biomass formed, however, they were much lower than the corresponding activities obtained during growth on xylan: the best transformants (ATX1-5 and ATX2-12) produced 5.1 and 5.0 U of XYN I and XYN II per g of biomass, respectively, which is considerably lower than the xylanase activity produced by the parent strain on xylan (13.2 U/g of biomass). Upon cultivation on xylan, the xylanase activities of strains ATX 1-5 and ATX 2-12 were only a little higher than those of the parental strain, which suggests that the *pki1* promoter is only poorly expressed on media not containing glucose.

TABLE 2. Specific xylanase activities of native and recombinant strains from this study during growth on glucose and on xylan[a]

Strain	Sp act (U/mg of protein), ± SD, during growth on:	
	Glucose	Xylan
TU-6 (*pyr4*$^+$)	ND	24 ± 5
ATX1-4	76 ± 18	25 ± 4
ATX1-5	68 ± 14	24 ± 5
ATX2-12	145 ± 27	30 ± 6
ATX2-16	118 ± 21	27 ± 5

[a] Activity assays were carried out after 36 and 48 h of growth on glucose and xylan, respectively.

Since the determination of xylanase activity in the presence

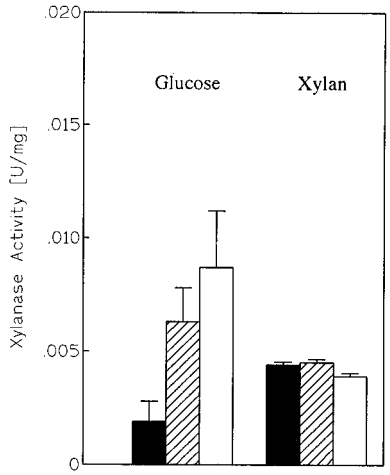

FIG. 4. Xylanase activity in cell extracts of *T. reesei* TU-6 (*pyr4* complemented [solid bars]), ATX1-4 (hatched bars), and ATX2-12 (open bars) upon growth on glucose (left) or xylan (right) for 36 and 72 h, respectively (n = 4).

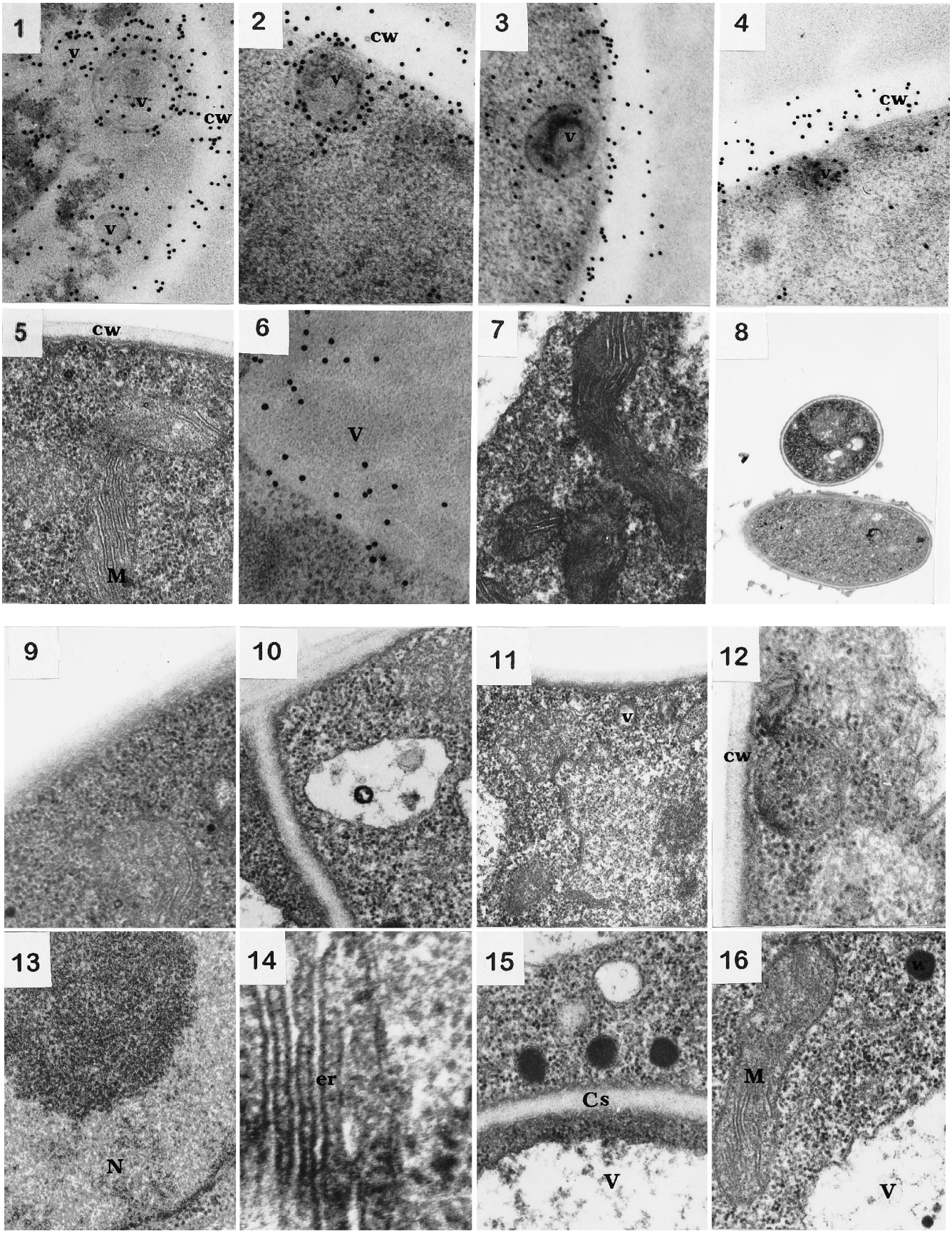
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FIG. 5. *T. reesei* TU-6 (*pyr4* complemented; pictures 7 to 12) and ATX2-12 (pictures 1 to 6 and 13 to 16), grown for 72 h in liquid medium (pictures 1, 2, 7, 8, and 13 to 16) or on solid medium (pictures 3 to 6 and 9 to 12) with glucose as the carbon source. The mycelium was fixed in glutaraldehyde. Samples were dehydrated by progressive lowering of the temperature and embedded at −35°C into LowicrylK4M (Chemische Werke Lowi, Waldkraiburg, Germany). Immunoelectron microscopic localization of XYN II was carried out on sections by immunogold labelling with a monoclonal antibody. Bound antibody was detected with goat anti-mouse immunoglobulin G–15-nm gold conjugate (Bio Cell, Cardiff, United Kingdom). Pictures 1 to 4 show cross and surface sections located at the periphery of young cells; picture 5 shows senescent cells from the central part of the colony; picture 6 shows vacuoles of senescent cells. Control samples (pictures 13 to 16), in which the ultrathin sections of *T. reesei* ATX2-12 were treated with mouse preimmunoserum followed by goat-anti mouse immunoglobulin G–15-nm gold conjugate, are also shown. Immunolabelling was also not observed in *T. reesei* QM 9414 growing on glucose (pictures 7 to 12). Abbreviations: cw, cell wall; cr, cross wall; N, nucleus; Nu, nucleolus; er, endoplasmic reticulum; v, vesicle; V, vacuole; M, mitochondrion; W, Woronin body. Magnifications, ×45,000.

of glucose from the medium was subject to interference by a high background, the enzyme activities formed on glucose were also measured by an alternative assay, which consisted of pipetting aliquots of enzyme solution onto agarose-xylan plates and staining the remaining xylan with Congo red after incubation (data not shown). Although the latter assay has a much higher standard deviation (±30%), the results were consistent with those from the reducing-sugar assay of xylanase activity. Finally, the intensity of the immunostaining supports the low xylanase secretion on glucose.

Intracellular accumulation of xylanase activity in *T. reesei* ATX1-5 and ATX2-12. To investigate whether the secretory capacity on glucose may contribute to the low activity found on glucose, we prepared cell extracts from *T. reesei* QM 9414, ATX2-12, and ATX1-5 grown on xylan and glucose, respectively, and measured the total xylanase activity. As shown in Fig. 4, the recombinant strains exhibited a clearly detectable intracellular xylanase activity, which was about twofold higher than that of the parent strain during growth on xylan. Since 1 g of biomass yielded 180 to 220 mg of extractable protein under the conditions used (depending on the strain), 1 g of biomass contains an average of about 2 U of xylanase activity, which is roughly a one-third of the total secreted activity. The intracellular xylanase activity of the parental strain on glucose was 0.0019 ± 0.0006 U/mg of protein, which is close to the detection limit of the assay. SDS-PAGE and Western blotting confirmed the presence of intracellular XYN I and XYN II, respectively, in the transformants during growth on glucose (data not shown).

Ultrastructural localization of XYN II for glucose-induced expression. To localize a possible limitation within the secretory pathway during growth of the recombinant strains on glucose, we examined the ultrastructural location of XYN II in strain ATX2-12 by using monoclonal antibody KA1 3.1 (34). Strain ATX1-5 was omitted from these studies, since the antibody available was not sensitive enough for use in immunoelectron microscopy. Figure 5 presents a selected sample of the pictures obtained. Particular care was taken to examine hyphal tips. Consistent with our previous observations (20), XYN II was immunologically detected in electron-dense vesicles located at the periphery of the cytoplasm and also in the cell wall of young hyphal tips (Fig. 5, picture 1). Senescent hyphal cells from the central part of surface-grown colonies show relatively less immunolabelling at the cytoplasmic periphery and the cell

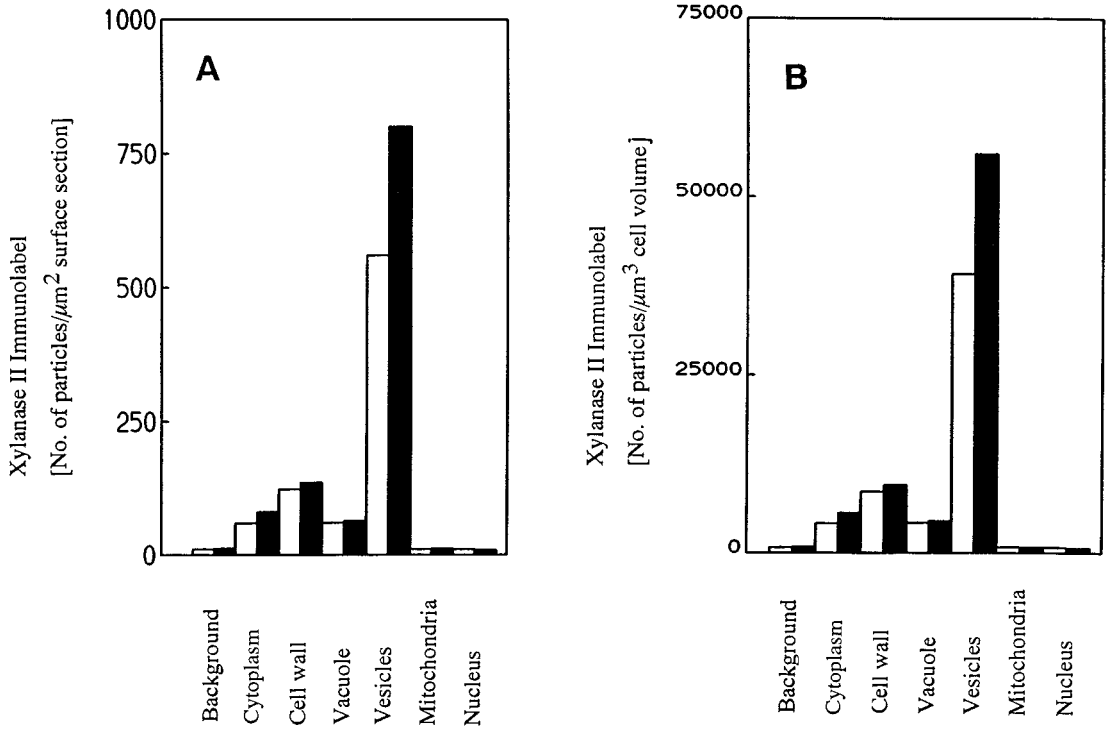

FIG. 6. Quantitative estimates of the immunolabelling of XYN II in organelles of *T. reesei* TU-6 (*pyr4* complemented) and ATX2-12, grown on xylan and glucose (72 h in liquid culture), respectively. The numbers of particles per square micrometer of surface section (A) and per cubic micrometer of cell volume (B) are given. Open bars indicate strain TU-6 (*pyr4* complemented); solid bars indicate strain ATX2-12. Means of 100 separate determinations are given, and the standard deviations are indicated by vertical bars.

wall; instead, XYN II was localized large vacuoles (Fig. 5, picture 2). No immunolabel was observed in the parent strain *T. reesei* QM 9414 on glucose. A lack of staining was also evident in control samples, in which mouse preimmunoserum had been used instead of monoclonal antibody KA1 3.1.

A dense labelling of XYN II was also observed in vesicles (Fig. 5, picture 3). When this labelling was compared with that of the parent strain, the density appeared to have increased in strain ATX2-12 (Fig. 6). Morphometric quantitation supported this observation, since roughly 1.4-fold more immunolabel was detected per vesicle number in this strain. In turn, the number of vesicles was about 1.3-fold decreased in strain ATX2-12. These findings were not the result of a general impairment in the pathway for secretion of xylanases in ATX2-12, since cultivation on xylan raised these figures to the range of values observed for strain QM 9414.

DISCUSSION

We have shown in this study that both xylanase isoenzymes of *T. reesei* can be produced under glucose-induced conditions. While the total xylanase productivity on glucose was only about one-third of that observed on xylan, the specific activities of the two xylanases were considerably increased because of the smaller amount of total proteins secreted during growth on glucose. Also, the selective overexpression of one of two isoenzymes will necessarily result in decreased total activities; when this fact is taken into consideration, the yields with XYN I, which usually accounts for about one-third of the total xylanase activity (35), are particularly promising. Inefficient glucose-induced secretion of proteins has been observed by several workers (26), yet the reasons for this finding are still matters of speculation. Most investigations have concentrated on potential bottlenecks at the level of transcription, mRNA stability, and proteolytic degradation (1, 17, 26, 37). The relative contribution of these steps is not known but probably varies with the organism investigated. We have therefore investigated in this study whether a secretory bottleneck may limit *T. reesei* protein secretion on glucose. We have observed the presence of a smaller number of secretory vesicles, albeit with a higher density of secretory enzymes (e.g., XYN II), during growth on glucose. We are unaware of the nature of these vesicles, but the observation of their fusion with the plasma membrane suggests a possible role in transport. We have also observed some increase in the amount and proliferation of the endoplasmic reticulum during growth on xylan. While we cannot completely rule out the possibility that these findings are due to mutation in strain ATX2-12 as a result of random integration of plasmid pAT5, we favor the interpretation that these differences are glucose specific. Ghosh and coworkers (9–12) documented that in the hypersecretory mutant *T. reesei* RUT C-30, an increased content of the endoplasmic reticulum is developed during growth on cellulose, and they interpreted this finding as explaining the increased rate of secretion of cellulases by this mutant. These authors also showed that this proliferation of the endoplasmic reticulum could be inhibited by the addition of glycerol, hence pointing to a possible repression of endoplasmid reticulum biosynthesis by some kind of carbon catabolite control in *T. reesei* (11). It is possible that such an effect also occurs at the level of vesicle formation.

The dissection of the yeast secretory traffic has revealed a number of genes involved in vesicular transport (4, 8), but even here, little is known about their regulation. Although our data do not provide evidence for an inefficient secretory pathway during growth of *T. reesei* on glucose, this topic deserves further attention if the secretion of proteins from monosaccharides and similar "repressing carbon sources" is desired.

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